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Title of Invention	[NEW COMPOSITION]

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Description

[NEW COMPOSITION]

BACKGROUND OF INVENTION

[0001] FIELD OF THE INVENTION

[0002] This invention relates to the field of atherosclerosis and ischemic cardiovascular diseases. In particular the invention relates to the field of pharmaceutical compositions comprising a phosphorylcholine conjugate for use as an immunogen, or the use of a monoclonal antibody with specificity to a phosphorylcholine conjugate, in the treatment or prevention of atherosclerosis in mammals having diagnosed signs of the disease. Furthermore, the invention also relates to the use of phosphorylcholine conjugates to produce a pharmaceutical composition optionally with an adjuvant. Furthermore the invention relates to diagnosing the presence or absence of IgM antibodies related to increased or decreased risk of developing ischemic cardiovascular diseases.

[0003] Atherosclerosis is a chronic disease that causes a thicken-

ing of the innermost layer (the intima) of large and medium-sized arteries. It decreases blood flow and may cause ischemia and tissue destruction in organs supplied by the affected vessel. Atherosclerosis is the major cause of cardiovascular disease including myocardial infarction, stroke and peripheral artery disease. It is the major cause of death in the western world and is predicted to become the leading cause of death in the entire world within two decades.

[0004] The disease is initiated by accumulation of lipoproteins, primarily low-density lipoprotein (LDL), in the extracellular matrix of the vessel. These LDL particles aggregate and undergo oxidative modification. Oxidized LDL is toxic and cause vascular injury. Atherosclerosis represents in many respects a response to this injury including inflammation and fibrosis.

[0005] In 1989 Palinski and coworkers identified circulating autoantibodies against oxidized LDL in humans. This observation suggested that atherosclerosis may be an autoimmune disease caused by immune reactions against oxidized lipoproteins. At this time several laboratories began searching for associations between antibody titers against oxidized LDL and cardiovascular disease. However, the

picture that emerged from these studies was far from clear. Antibodies existed against a large number of different epitopes in oxidized LDL, but the structure of these epitopes was unknown. The term "oxidized LDL antibodies" thus referred to an unknown mixture of different antibodies rather than to one specific antibody.

[0006] It is well established that there is an ongoing inflammation in the atherosclerotic lesions, characterized by activation of immune competent cells and production of inflammatory cytokines. Established risk factors like hypertension, blood lipids, diabetes and smoking are likely to promote this inflammatory reaction, but the mechanism by which this occurs are not well characterized and different non-mutually exclusive possibilities exist. Several different autoantigens that could elicit this immune reactivity have been proposed, including oxidized low density lipoprotein (oxLDL) and heat shock proteins (HSP)[2,3]. Available data on the role of immune reactions in atherosclerosis indicate a complex relationship. One example of this is immunization in animal models to influence atherogenesis. When HSP is used, atherosclerosis increases but decreases when oxLDL is the antigen[4,5].

[0007] The role of aOxLDL in human disease appears to be com-

plex. In humans, it has previously been demonstrated that aOxLDL is higher in healthy controls than in men with borderline hypertension, an example of early cardiovascular disease[6]. Recent studies are in line with this observation[7,8]. On the other hand, several authors have reported that aOxLDL are raised in human cardiovascular diseases (CVD), especially at later stages[2,3,9,10.] One example is systemic lupus erythematosus (SLE) and autoimmune disease associated with a very high risk of CVD. SLE-patients with a history of CVD had clearly raised aOxLDL levels[11]. These to some extent contradictory results may depend on different methods and stages of LDL-oxidation, yielding differences in antigenicity. It is also likely that disease stage and risk factor profile are related to antibody levels.

[0008] Oxidized low density lipoprotein (oxLDL) itself has many proinflammatory properties including activation of T cells [12,13], monocytes/macrophages and endothelial cells[14-16]. OxLDL promotes inflammation also in immune competent cells from atherosclerotic lesions[17]. However, it should be noted that oxLDL may also ameliorate acute inflammatory reactions and instead promote a more low-grade chronic inflammation as that seen in atherosclerosis[18]. It is interesting to note that many biological effects of oxLDL are

caused by platelet activating factor (PAF)-like lipids in oxLDL[19-21].

[0009] Phosphorylcholine (PC) is a major component not only in inflammatory phospholipids like platelet activating factor-PAF (where it is essential for interaction with the PAF-receptor) and in oxLDL, but is also as an immunogenic components of many bacteria including S. Pneumoniae[22]. Furthermore, PC is expressed by apoptotic cells[2,23].

[0010] In US5455032 phosphocholine conjugates have been used in vaccines for inducing immunoprotection against infections such as Streptococcus pneumoniae. In a recent study [24] by Binder et al on pneumococcus vaccine in mice, it was also shown that vaccination decreased atherosclerotic lesion formation. It was found that many autoantibodies to oxLDL derived from atherosclerotic mice share structural identity with antibodies which protect against common infectious pathogens, including Streptococcus pneumoniae. The study does not give any information about specificity, or that IgM anti-phosphorylcholine antibodies are significantly more important than corresponding IgG antibodies as a protecting factor in atherosclerosis. Furthermore, phosphorylcholine conjugates have not been used in the pneumococcus vaccine.

[0011] In another study it was shown [25] that antiphosphoryl-choline antibody levels are elevated in humans with periodontal diseases. The conclusion is that phosphoryl-choline is an important oral antigen associated with organisms in the periodontal flora and that anti-PC antibody is elevated as a consequence of periodontal disease. No information is given with regards to the antibodies and possible protection of or progression of atherosclerosis.

[0012] A couple of documents (e.g. WO2002080954 and WO0168119) related to immunization treatment of atherosclerosis have been published but these are either based on the use of peptide fragments of apolipoprotein B or antibodies to alpha/beta chains of a T cell receptor. A method to detect atherosclerotic plaque (WO9908109) using monoclonal antibodies to oxidation-specific epitopes on lipoprotein has also been described. This is different from the method proposed in this invention where a phosphorylcholine conjugate is used to detect IgM antibodies in subject samples.

SUMMARY OF INVENTION

[0013] The invention relates to pharmaceutical compositions comprising a phosphorylcholine conjugate or a monoclonal antibody with specificity to a phosphorylcholine

conjugate and the use of these compositionsin the treatment or prevention of atherosclerosis. Furthermore, the invention also relates to the use of phosphorylcholine conjugates or said monoclonal antibody to produce a pharmaceutical composition optionally with an adjuvant. With phosphorylcholine conjugate means a phosphorylcholine moiety linked to a carrier, preferably via a spacer. The structural element phosphorylcholine also comprises derivatives of phosphorylcholine. The invention also relates to methods to determine the presence or absence of IgM antibodies against phosphorylcholine which are related to an increased or decreased risk of developing ischemic cardiovascular diseases. The invention is further described below.

DETAILED DESCRIPTION

[0014] The examples disclosed below are provided only for the purpose of illustrating the present invention and should not be considered as any limitation of the scope as outlined in the appended claims.

[0015] An example of a method to determine the presence or absence of IgM antibodies against phosphorylcholine which is related to an increased or decreased risk of developing ischemic cardiovascular diseases is described. Other

methods known in the art can also be used.

[0016] Methods to determine the presence or absence of IgM antibodies against phosphorylcholine

[0017] IgM antibodies to PC-BSA were determined by an enzyme-linked immunsorbent assay method.

[0018] A microtiter plate was coated with PC-BSA (10µg/ml) in phosphate buffered saline (PBS). After washings with PBS , the plates were blocked with a 2% BSA solution. Serum samples were diluted (1:30) in 0.2% BSA-PBS . Plates were incubated overnight at 40°C and washed. Alkaline phosphatase conjugated goat anti-human IgM (diluted 1:7000 in the sample buffer) were added at 100ul/well and incubated at 40°C overnight. After washings, colour was developed by adding an alkaline phosphatase substrate and incubating the plates for 60 min at room temperature in the dark. The absorbances were read in a spectrophotometer at 405nm.

[0019] Different carriers and spacers for phosphorylcholine have been tested. The exemplified carriers are not limited to these. Other carriers such as other proteins, lipids or polymers, such as latex beads which are known in the art, may also be used.

[0020] Synthesis of a phosphorylcholine conjugate and prepara-

tion of a pharmaceutical composition

[0021] Latex beads (0.20 µm or 0.81µm) were suspended in PBS and mixed over night with a 10 µg/ml solution of phosphorylcholine-BSA. The beads were then centrifuged and washed several times with buffer and blocked with a 10µg/ml solution of BSA. After another repeated washing, the beads were resuspended to a suitable concentration in a suitable buffer and stored refrigerated until use.

[0022] Phosphorylcholine with a linker arm can also be conjugated to KLH (keyhole limpet hemocyanin) via a diazophenyl group. More preferably a p-nitrophenyl-6-(O-phosphocholine)hydroxyhexanoate derivative of PC can be synthesized according toChesebro, B. and Metzger, H. (1972) Biochem. 11:776. p-Nitrophenyl-6-(O-phosphocholine) hydroxyhexanoate was dissolved in dry acetonitrile (100 mg/ml) just prior to adding it to the KLH. Derivative and KLH were mixed overnight at 4° C and then dialyzed to remove unbound spacer and p-nitrophenylate, which is the leaving group.

[0023] An injection solution of the prepared phosphorylcholine conjugate, suspended in a suitable buffer, can be directly used for immunization.

[0024] Immunization with a phosphorylcholine conjugate

[0025] A high titer of IgM antibodies recognizing phosphoryl-choline was determined in plasma from BALB/c mice after immunization with 200 µg [p-Nitrophenyl-6-(O-phosphocholine) hydroxyhexanoate KLH] i.p. using the suggested immunoassay method.

[0026] Monoclonal antibodies against a phosphorylcholine conjugate

[0027] Monoclonal antibodies can be produced using any standard method known in the art. See for example "Briles DE, Forman C, Hudak S, Claflin JL. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J Exp Med. 1982;156:1177-85"or "T15 PC binding monoclonal antibodies retain specificity when they switch from IgM to IgG. , Spira, Gad; Aguila, Hector L.; Scharff, Matthew D. Fac. Med., Techniton-Israel Inst. Technol., Haifa, Israel. Journal of Immunology (1988), 140(8), 2675-80".

[0028] IgM immunoglobulin levels in atherosclerotic subjects

[0029] IgM autoantibody levels against phosphorylcholine in subjects with hypertension (diastolic pressure>95 mmHg) were determined at baseline and after 4 years in a correlation study of risk factors for atherosclerosis. The results are summarized below.

[0030] Carotid plaques were detected in 77 subjects (35%) at enrolment, and in 84 subjects (38%) at the 4-year follow-up. In total 218 human subjects were in the study. Increases in intima-media thickness (IMT) at follow-up were less prevalent in subjects having high serum levels of IgM to PC (75^{th} or 90^{th} percentile) at the time of enrolment. There is a significant difference between mean values in IgM anti-phosphorylcholine antibody levels between individuals with increased and decreased IMT (638.8±219.6 vs. 734.8±266.9, p=.004).

[0031] The relationships between IgM autoantibodies to PC and changes in IMT were independent of age, smoking habits, treatment with atenolol or lacidipine and blood lipids. IgM autoantibodies were also independent of IgG values.

[0032] One embodiment of the present invention is thus to use a phosphorylcholine conjugate for the preparation of a pharmaceutical composition to be used in the treatment or prevention of atherosclerosis. The conjugate can be phosphorylcholine linked to a protein or to a polymer. The pharmaceutical composition is preferably given by injection.

[0033] The proposed method of active immunization will modulate the autoantibodies titer which in turn will have a posi-

tive effect on the development of atherosclerosis.

[0034] Another embodiment of the invention is to use a monoclonal antibody recognizing a phosphorylcholine conjugate for the preparation of a pharmaceutical composition to be used in the treatment or prevention of atherosclerosis. The monoclonal antibody can be produced using methods known in the art.

[0035] A further embodiment of the invention is to provide a method of diagnosing the presence or absence of IgM antibodies towards phosphorylcholine which factor is related to an increased or decreased risk of developing ischemic cardiovascular diseases, using a phosphorylcholine conjugate. A preferred method is an immunoassay.

[0036] EXPERIMENTAL

[0037] Subjects

[0038] Serum samples were obtained from 226 subjects with established hypertension (diastolic pressure >95 mm Hg) prior to their entry into the Swedish component of the European Lacidipine Study on Atherosclerosis (ELSA)[25,26]. Samples were collected following a 4-week washout period with no medication to minimize the effects of treatment on the measured parameters. Blood pressure,

cholesterol and triglyceride levels were determined as described previously[25,26]. One hundred and fifteen of the subjects were subsequently assigned to treatment with the β-blocker atenolol, and 111 of the subjects were assigned to treatment with the calcium antagonist lacidipine. The study was approved by the Ethics Committee of the Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

[0039] Carotid ultrasound

[0040] Carotid ultrasound determinations were performed and analysed as detailed elsewhere25,26. A total of 218 patients had valid ultrasound measurements at baseline and 4-year follow up. Briefly, the right and left carotid arteries were examined with Biosound 2000 IIA duplex scanner using an 8.0 MHz annular array transducer. The intima-media (I-M) thickness was determined in the far wall as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitious echo. The outcome measurement as a surrogate indicator for atherosclerosis was the change in mean maximum Intimal-Medial thickness (IMT) of the four far walls in the distal common carotids and carotid bifurcations bilaterally

(CBMmax) at the 4-year follow-up. The associations between antibody levels to PC at enrolment into the study with an increase or decrease in IMT at the 4-year follow-up were evaluated.

[0041] Reagents

[0042] Polysorp F96 microtiter immuno-plates were purchased from Nunc (Roskilde Denmark), PC-BSA (Phosphorylcholine-Bovine Serum Albumin) was purchased from Biosearch Technologies, INC (USA). Bovine serum albumin (BSA), Alkaline phosphatase conjugated goat anti-human IgG(r-chain specific) , Alkaline phosphatase conjugated goat anti-human IgM(u-chain specific), PNPP(Alkaline phosphatase substrate), were obtained from Sigma (St. Louis, MO, USA).

[0043] Specificity of anti-phosphorylcholine-BSA antibodies

[0044] In order to investigate the specificity of anti-phosphorylcholine-BSA, absorption assays were performed by use of pooled high titer sera. At a dilution giving 50% of maximal binding to PC-BSA, high titer pooled sera were preincubated with different concentration of PC-BSA, After vortexing, the tubes were incubated at 40°C overnight and centrifuged at 13000 r.p.m. for 30

min(40°C) The supernmatants were tested for antibody binding to PC-BSA as described.

[0045] Statistical analysis

[0046] Anti-phosphorylcholine levels were dichotomized at the 75th and 90th percentile. The association between anti-phosphorylcholine and the progression of atherosclerosis over a 4-year period were determined by estimating increases in IMT (yes or no) using logistic regression analysis and the calculation of odds ratios (ORs) and 95% confidence intervals (CI). Adjustments were made for possible confounders including age, smoking habits, serum cholesterol, serum triglycerides and mode of anti-hypertensive treatment (lacidipine, atenolol). A two-tailed p-value < 0.05 was considered as significant.

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Claims

[c1] 1.A pharmaceutical composition comprising at least one phosphorylcholine conjugate or a monoclonal antibody with specificity to a phosphorylcholine conjugate, intended for immunization and treatment of mammals, including humans, against atherosclerosis or atherosclerotic related diseases and said immunization having immunogenic or therapeutic properties against atherosclerosis.

[c2] 2.The pharmaceutical composition according to claim 1, wherein said composition is administered by injection.

[c3] 3.Conjugates according to claim 1 and 2, wherein the phosphorylcholine is linked to a carrier via a spacer.

[c4] 4.Conjugates according to claim 3, wherein the carrier is a protein.

[c5] 5.Conjugates according to claim 4, wherein the protein is KLH (keyhole limpet hemocyanin) or human serum albumin (HSA).

[c6] 6. Conjugates according to claim 3, wherein the carrier is latex beads.

[c7] 7.The use of one or more of the phosphorylcholine conjugates in claim 1-6, in the preparation of a pharmaceutical composition, optionally in combination with an adjuvant, intended for immunotherapy or therapy for the treatment of ischemic cardiovascular diseases.

[c8] 8.Method of prophylactic or therapeutic treatment of a mammal, including a human being, suffering from atherosclerosis or facing the risk of developing ischemic cardiovascular diseases, whereby a therapeutically effective amount of at least one phosphorylcholine conjugate or a monoclonal antibody with specificity to a phosphorylcholine conjugate is administrated.

[c9] 9.Method of diagnosing the presence or absence of IgM antibodies related to increased or decreased risk of developing ischemic cardiovascular diseases, using a phosphorylcholine conjugate.

[c10] 10.Method according to claim 9 wherein phosphorylcholine is linked to a carrier via a spacer.

[c11] 11.Method according to claim 10 wherein the carrier is a protein

[c12] 12.Method according to claim 11 wherein the protein is KLH (keyhole limpet hemocyanin) or human serum albu-

min (HSA).

[c13] 13.Method according to claim 10, wherein the carrier is latex beads.

[c14] 14.Method according to claim 9-13, wherein the assay is an immunoassay.

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[NEW COMPOSITION]

Abstract

IgG and IgM autoantibody levels against phosphorylcholine in subjects with hypertension (diastolic pressure>95 mmHg) were determined at baseline in order to determine the importance of antibodies for the development of atherosclerosis. The results show that increases in intima-media thickness (IMT) at a follow-up four years after baseline were significantly less prevalent in subjects having high IgM autoantibodies to phosphorylcholine. The presence or absence of IgM autoantibodies against phosphorylcholine is thus related to an increased or decreased risk of developing ischemic cardiovascular diseases. A method to determine IgM antibodies toward phosphorylcholine is proposed in this invention to identify subjects at risk of developing ischemic cardiovascular diseases. Animal experiments show that medium to high levels of IgM antibodies can be detected in plasma after active immunization with a keyhole limpet hemocyanin (KLH)-phosphorylcholine conjugate. A pharmaceutical composition comprising a phosphorylcholine conjugate (active immunization) or a monoclonal antibody with specificity to a phosphoryl-

choline conjugate (passive immunization) is proposed and the use of these compositions as active or passive immunogens in the treatment or prevention of atherosclerosis.